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13. SUPPLEMENTARY NOTES

14. ABSTRACT

In the past year we have progressed substantially towards completion of our project goals. We have redesigned our lentiviral protocol for restoration of AIF protein expression (and variants thereof) in shAIF ablated PC3 cells. This has allowed the construction of a panel of PC3-derived cells lines that either overexpress AIF variants (wildtype or enzymatically inactive TVA) or express these variants in the absence of endogenous AIF protein. This panel of cells was subjected to 3 dimensional culture experiments, which indicated that whereas suppression of AIF in these cells abrogated 3 dimensional growth, restoration of expression with wildtype AIF, but not the TVA variant, was critical to supporting normal growth in 3 dimensional cultures. Molecular analysis of these cells indicated that AIF suppression led to a decrease in the expression of complex I of the mitochondrial electron transport chain, and that either wildtype or TVA-AIF could restore this expression. Interestingly, despite normal complex I levels, TVA expressing cells exhibited high levels of glycolysis, similar to AIF deficient cells, suggesting that the glycolytic switch observed upon AIF ablation is related to enzymatic activity of the protein. Overall these data show for the first time that the ability of AIF to support prostate cancer cell growth is dependant upon the enzymatic activity of the protein.

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Introduction

As the second most common form of cancer diagnosed among men in the western world prostate cancer represents a significant healthcare threat, and there is great need for new and more effective treatments for this disease. This research project seeks to determine the potential role of two proteins, apoptosis inducing factor (AIF) and X-linked inhibitor of apoptosis (XIAP), in prostate cancer pathogenesis. Both proteins are multifunctional, and one of our goals is to determine which properties of each molecule are important for disease progression. This report summarizes progress made in the past year towards completing these research goals.

Body

We have made significant progress towards completing the tasks outlined in our original Statement of Work, as summarized below:

Task 1b. To determine the specific properties of AIF and XIAP required for tumorigenesis.

- **Generation of PC3-derived "restoration" cell lines expressing AIF and XIAP variants lacking defined functions (Months 10-18).** As described in our last two annual reports we have succeeded in producing lentiviruses containing sequences to express wildtype AIF and the AIF variants T263A/V300A (TVA mutant) and K255/265/510/518A (K4A mutant). For reasons that were unclear, we had been unsuccessful at using these lentiviruses to stably infect AIF deficient cells in order to generate "restoration" cells lines with these AIF variants. In the past year we have changed the lentiviral backbone used for the production of lentivirus to a smaller construct than described in our previous efforts, and this new approach has proven successful for the establishment of PC3-derived cells that express the wildtype and TVA variant described above. Immunoblot analysis indicated restored expression is shown in Figure 1. Interestingly, we have been unable to achieve expression of the K4A mutant, likely due to toxicity associated with stable expression of this protein. As our hypothesis remains that the enzymatic activity of AIF is more important than death induction, we have proceeded without the K4A variant in our subsequent experiments.
- **Assessment of the PC3-derived tumor progression (Months 18-27).** We are currently preparing the cell lines described above of in vivo xenograft experiments to test the ability of "restored" cell lines to exhibit tumorigenic growth. In preface to these studies, we have examined the ability of these cell lines to grow in three-dimensional cell culture experiments, which indicate that only the wildtype AIF protein, and not the TVA variant, is capable of supporting normal three dimensional growth of shAIF-PC3 cells (Figure 2).

Task 2. To investigate the contribution of AIF to tumorigenesis in a transgenic murine model of cancer**.**

- **Breed Pten$^{-/-}$ with AIF-deficient (Hq) mice (Months 24-30).** Experiments are in progress.

- **Evaluation of tumor progression/burden in Pten$^{-/-}$/Hq mice (Months 30-36).** Experiments are in progress.

In addition to the progress described above relating to the original statement of work, we have made additional progress related to understanding the role of XIAP and AIF in the control of prostate cancer pathogenesis. Stemming from our observations shown in Figure 2, that the enzymatic activity of AIF is necessary for tumorigenic growth, we have assess the affects of AIF

ablation upon expression of the mitochondrial electron transport chain component, complex I (Figure 3). Ablation of AIF alone in PC3 cells leads to a substantial reduction in complex I protein levels when compared to controls. Interestingly, and in contrast to our three-dimensional growth experiments, the TVA variant is able to restore normal complex I expression to shAIF cells. However, when glucose consumption was assessed, we noted that whereas AIF ablated cells display substantially higher levels of glucose consumption, only wildtype AIF and not the TVA variant was able to restore glucose consumption back to control levels (Figure 4).

An open question related to the overall activity of AIF in prostate cancer cells was whether the death inducing capabilities of AIF had a role to play in cancer progression. To answer this question, we assessed the ability of the DNA alkylating agent MNNG, reported by previous studies to induce an AIF-dependent cell death pathway in a variety of other cell types, to induce cell death in PC3 cells either lacking AIF, or overexpressing either wildtype or TVA AIF. As shown in figure 5, neither the ablation nor overexpression of AIF in PC3 cells resulted in a change in sensitivity to MNNG treatment.

Key Research Accomplishments

- Restoration/overexpression of both wildtype and AIF-TVA has been achieved in our panel of PC3 cell lines
- Matrigel growth of PC3 cells as a consequence of AIF protein expression was assessed
- The necessity of AIF for maintenance of complex I protein levels in PC3 cells was determined
- The impact of AIF protein expression upon glucose consumption was assessed
- The lack of AIF dependence to MNNG-induced cell death was definitively shown

Reportable Outcomes

None to date

Conclusions

Our current conclusions are 1) The enzymatic activity of AIF is necessary for PC3 cells to growth in three dimensional culture, 2) complex I expression requires AIF but is not dependant upon AIF enzymatic activity, 3) glucose consumption is elevated following AIF ablation, only wildtype AIF is capable of restoring glucose consumption back to normal levels, and 4) AIF plays no role in controlling the death of PC3 cells following treatment with the DNA alkylating agent MNNG. Overall these data shown for the first time that PC3 cells required the enzymatic activity of AIF for normal energy metabolism and growth in three dimensional culture. Studies currently in progress are designed to determine which properties of AIF are necessary for support of tumor growth in vivo.

References

NA

Supporting Data

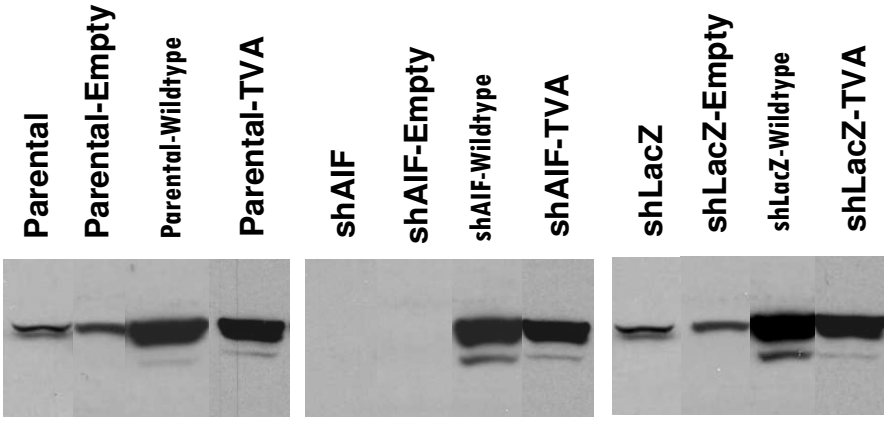

Figure 1. Restoration of AIF in shAIF-PC3 cells, overexpression in control cell lines. Parental (left), shLacZ (right) and shAIF (center) PC3 cells were stably infected with either a control lentivirus (empty) or lentiviruses containing cDNA encoding either wildtype AIF or the enzymatically deficient AIF variant TVA. Stable integration was selected by growth in puromycin. After selection, cells were subjected to immunoblot analysis to determine AIF expression levels. Note overexpression of wildtype and TVA-AIF in parental and shLacZ cells, and restoration of expression in shAIF cells. Equal loading of protein lysates was determined by immunoblot analysis (data not shown).

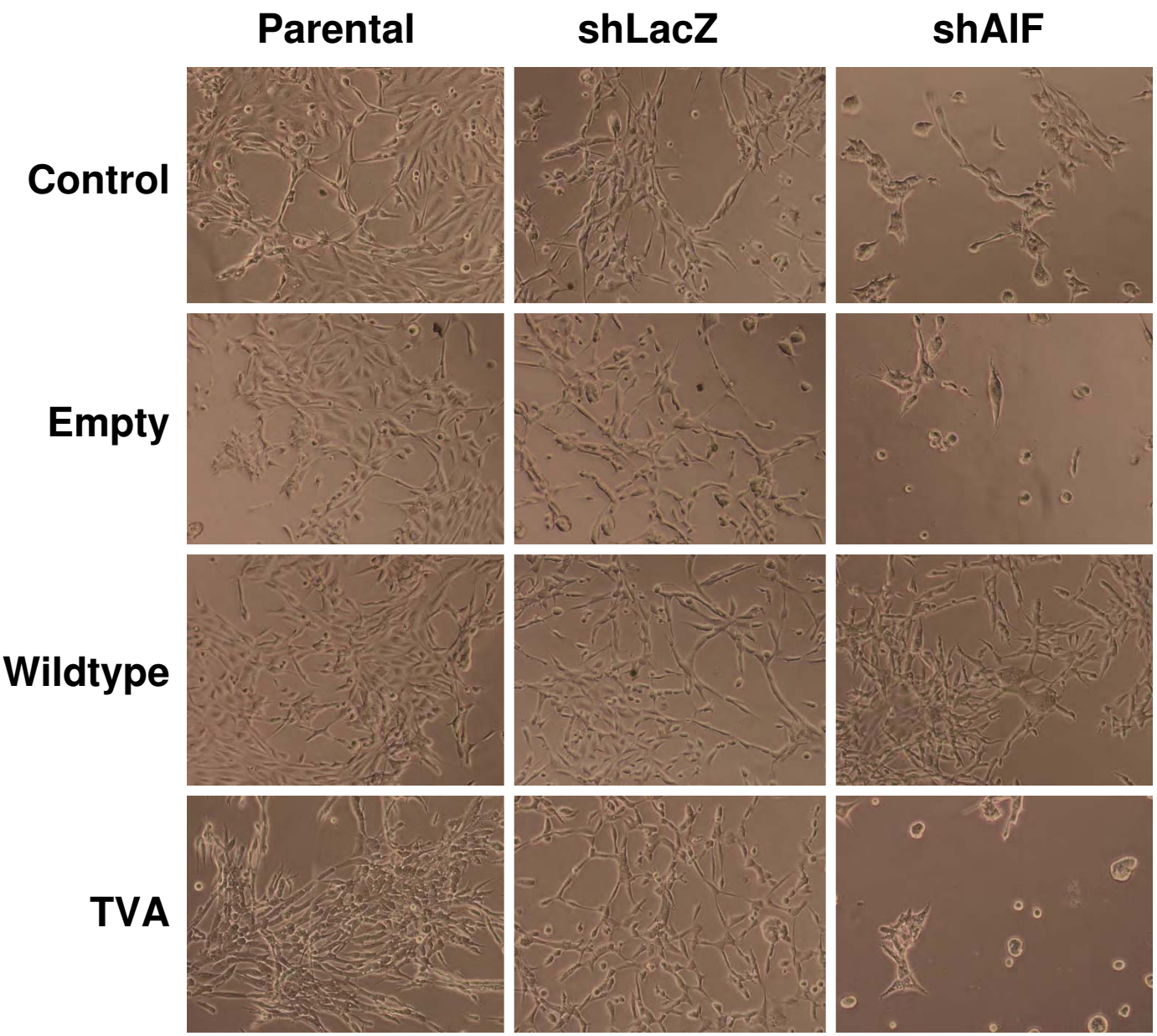

Figure 2. Matrigel growth of AIF-Deficient PC3 is restored by wildtype but not enzymatically deficient AIF. Parental (left column), shLacZ (center column) and shAIF PC3 cells (right column) restored infected with lentiviruses encoding the indicated AIF variants were plated at 10,000 cells per well in 12-well culture dishes coated with a layer of Matrigel basement membrane. Cells were allowed to grow for 96 h, and then morphological features of cell growth were assessed by phase contrast microscopy (magnification 10X). Note that shAIF cells (control, empty) were unable to grow in matrigel compared to parental and shLacZ cells, and that only restoration of shAIF cells with wildtype AIF was capable of allowing shAIF cells to grow. Further note that overexpression of either wildtype or TVA-AIF in parental and shLacZ cells had no observable impact on matrigel growth.

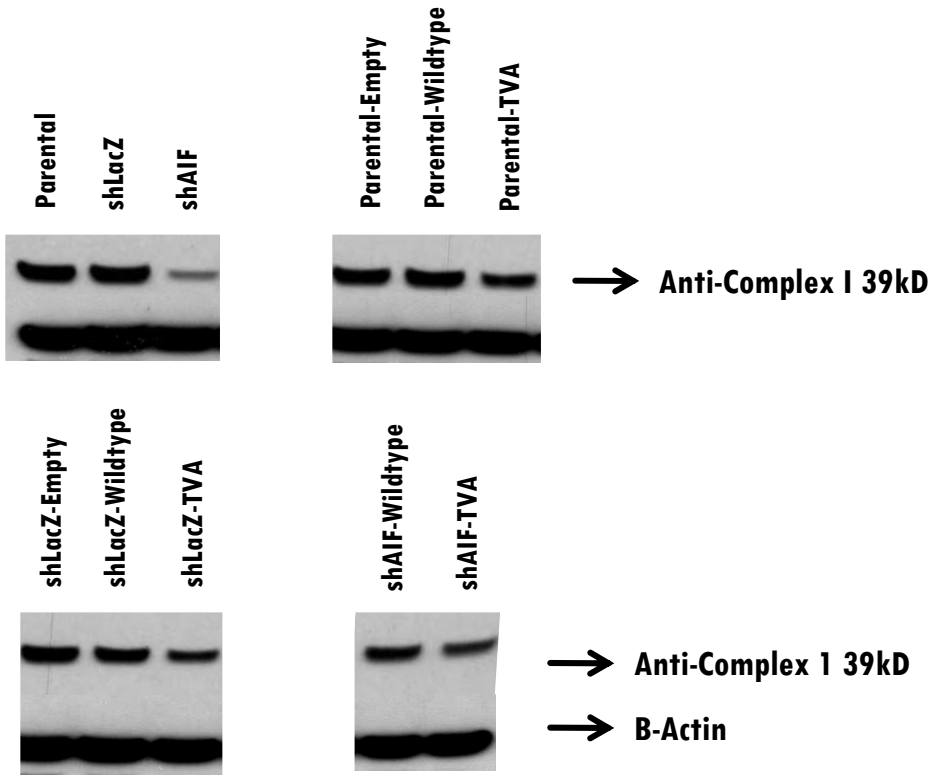

Figure 3. The 39 kDa subunit of Complex I is sensitive to AIF expression but not eznymatic activity. Whole cell lysates from derivatives of parental (top row) and both shLacZ and shAIF PCS cells (bottom row) were subjected to immunoblot analysis for the presence of the 39 kDa subunit of mitochondrial Complex I of the energy transport chain. Whereas AIF ablation in control shAIF cells leads to a substantial decrease in complex I (top row, left panel), restoration of these cells with either wildtype or TVA-AIF is sufficient to restore expression to normal levels (bottom row, right panel). Interestingly, whereas overexpression of wildtype AIF has no impact on complex I levels in parental or shLacZ cells, the expression of the TVA variant in these cells led to a slight suppression in complex I levels, suggesting that TVA may function as a dominant negative protein in these lines.

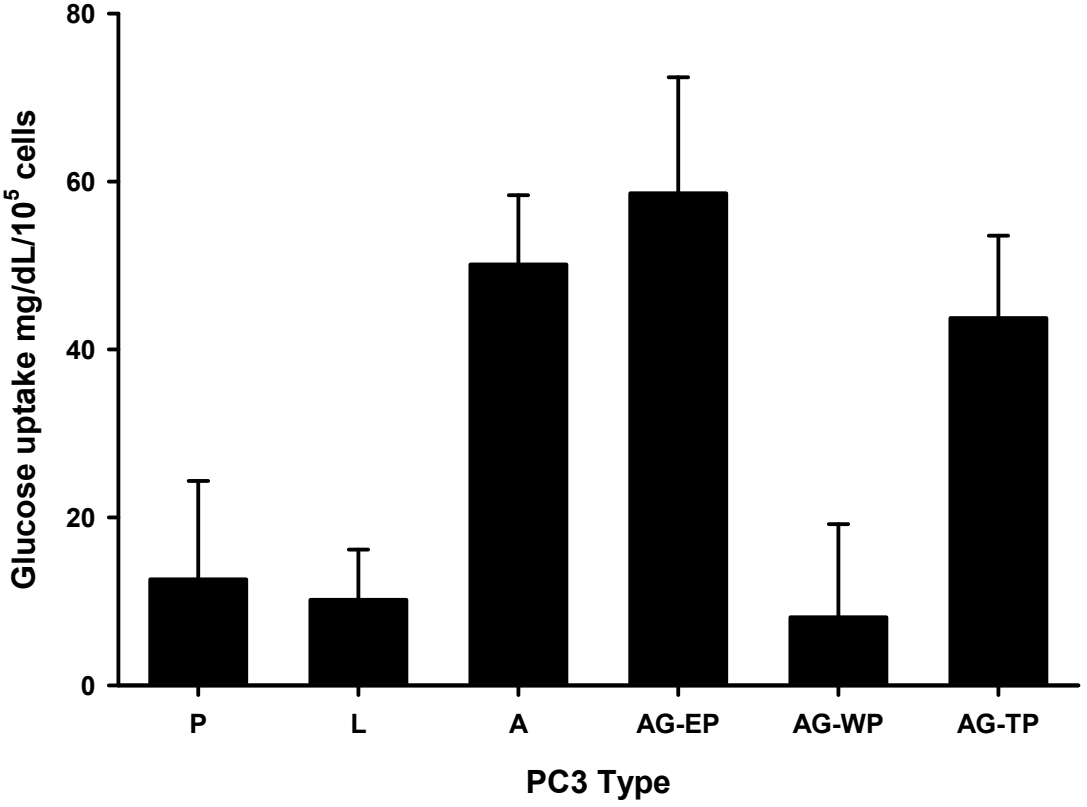

Figure 4. Glycolytic switch in AIF-Deficient PC3 cells is reverted only by restoration of enzymatically active AIF. Parental (P), shLacZ (L), shAIF (A), shAIF+empty (AG-EP), shAIF+WT (AG-WP), and shAIF+TVA (AG-TP) cells were seeded in 6-well plates and allowed to grow for 48h. Growth media was collected and the amount of glucose remaining was determined. Total cell number was determined by Coulter counting, and glucose consumption per cell was calculated. Note that AIF suppression in shAIF cells leads to a substantial increase in glucose consumption, consistent with an energetic switch to glycolysis for energy production, rather than oxidative phosphorylation. Also note that only wildtype AIF restored glucose consumption to normal levels; the shAIF cells expressing the TVA variant remained highly glycolytic.

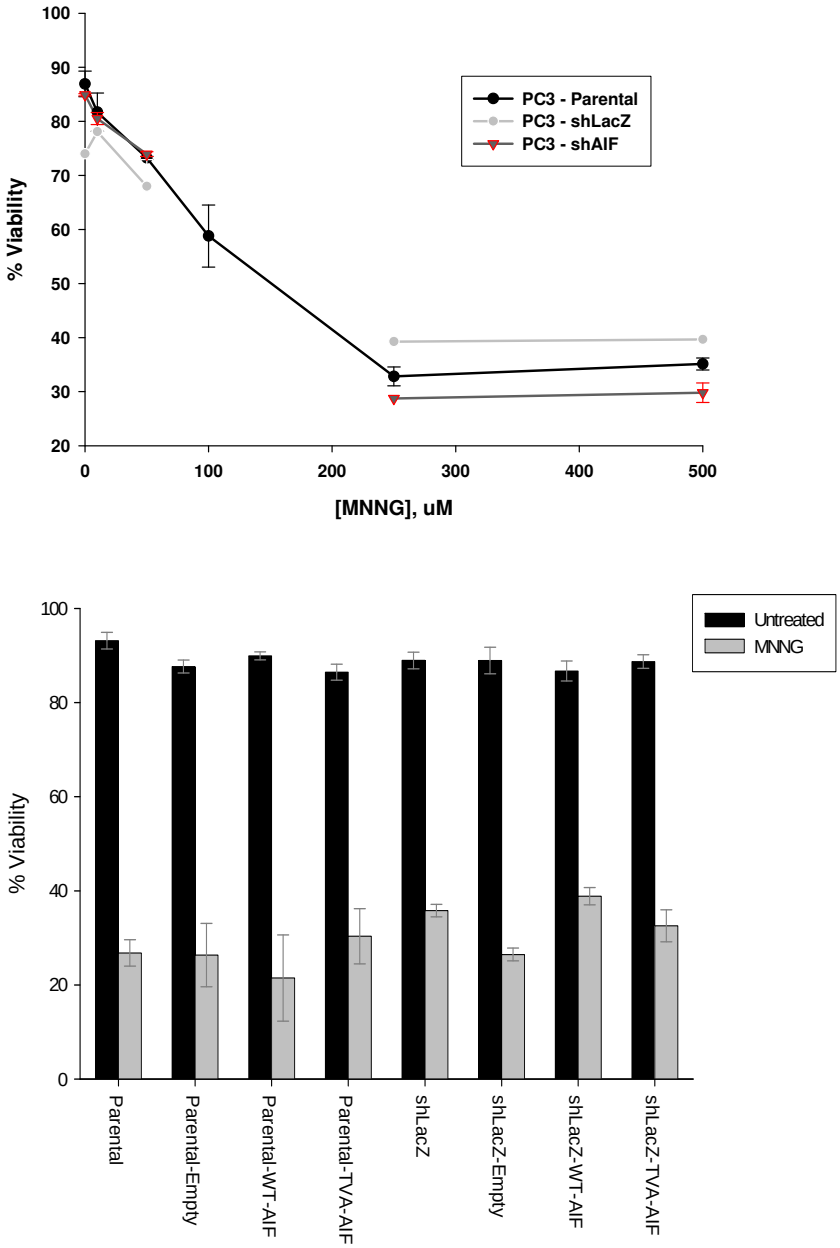

Figure 5. AIF is not involved in cell death mediated by the DNA alkylating agent MNNG. Top: Parental, shLacZ, and shAIF cells were treated with increasing concentration of MNNG for 15 minutes, drug was removed, and cells were incubated for an additional 20 h prior to determination of viability by propidium iodide staining and flow cytometry. Note that AIF ablation had no effect on the sensitivity of cells to MNNG treatment. Bottom: Parental and shLacZ cells overexpressing wildtype or TVA AIF were left untreated (black bars) or treated with MNNG (500 uM, gray bars) and analyzed as described above. Note that overexpression of neither wildtype nor TVA AIF affected the death or survival of parental and shLacZ cells.